Surface-conjugated earthworm mega-hemoglobin as an oxygen therapeutic

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1 Abstract

Emergency medical applications involving blood loss frequently require donated blood to enhance oxygen delivery to tissues and restore blood volume in a patient. However, donated blood is in short supply and has complications related to type-matching and blood-transmitted diseases. These limitations have inspired the development of red blood cell alternatives also known as hemoglobin-based oxygen carriers (HBOC). Naturally extracellular hemoglobins, such as that of the earthworm (LtEc), avoid side effects such as extravasation and systemic hypertension associated with transfusion of mammalian derived HBOCs due to their larger size and higher structural stability in the bloodstream. This work focused on surface coating LtEc with oxidized dextran (Odex), an inexpensive, biocompatible polysaccharide, to overcome the potential immune response *in vivo*. Dextran was functionalized via oxidation then reacted with pure LtEc protein to form Odex-LtEc. Compared to native LtEc, Odex-LtEc showed increased size and molecular weight. Odex-LtEc also had a higher oxygen affinity, slower oxygen offloading rate, and a higher rate of autoxidation. Although certain HBOC characteristics such as increased size are linked to higher stability, animal model trials will be necessary to determine the efficacy of Odex-LtEc *in vivo.* Reaction conditions may also be further optimized to find a suitable amount of surface coating to retain desirable HBOC characteristics while improving structural stability.

2 Introduction

The American Red Cross estimates that 36,000 red blood cell units are required in the U.S. every day.[1] With a shelf life of 42 days and type-matching requirements, donated blood is in short supply compared to the demand of these patients. Blood also needs to be refrigerated, which can limit its portability to remote areas such as combat zones. While several generations of hemoglobin-based oxygen carriers (HBOCs) have used mammalian hemoglobin (Hb) as a starting point for blood substitutes, these products have been correlated with toxic effects in clinical trials.[2] Several issues were linked to the small size of mammal hemoglobin, which allows it to tunnel into the endothelial lining and scavenge vasoregulatory molecules.[3–5] Researchers have been investigating larger HBOCs to mitigate these effects, and several HBOCs have been developed from intermolecular binding or polymerization of mammal HBOCs to increase their size.[6] However, the specificity of reaction conditions and large size distribution of product complicate the creation of these HBOCs.[7]

Hemoglobins from the annelid phylum, including that of the earthworm L. terrestris (LtEc protein), have garnered attention as naturally large, stable oxygen-carrier proteins.[8] Occurring in the coelomic fluid of the worm, LtEc is highly stable and less prone to dissociation and denaturation than mammalian Hbs.[9] Unlike Hbs of mammals, LtEc does not rapidly scavenge nitric oxide, a reaction linked to hypertension in cell-free hemoglobin transfusions. LtEc is also less prone to oxidation of the heme group than extracellular mammal Hb due to embedded stabilizing ions, allowing it to retain oxygen-carrying capability for a longer time.[10] Palmer et al. have developed a scalable purification method to isolate ultrapure LtEc, making it more feasible to collect protein for *in vivo* studies and potential therapeutic use.[11]

Although LtEc is naturally stable, studies have been performed to modify the protein. Since native LtEc is produced by annelids, it is quickly cleared from circulation by mammal immune systems. Conjugating biocompatible materials to the surface of HBOCs allows a longer transfusion half-life due to the camouflage provided by the material adhering to the protein. Modification of LtEc via conjugation to functionalized polyethylene glycol (PEG), a highly hydrophobic molecule, was shown to produce a stable molecule at a specific reaction ratio of PEG to LtEc.[12] The PEG-LtEc also was retained longer in circulation than native LtEc. However, due to anti-PEG antibody creation in certain patients, alternative surface coatings should be investigated.[13] Dextran is a polysaccharide widely used as a plasma expander and can be functionalized via oxidation. The resulting aldehyde groups can then be bonded to amines in protein, covering the surface of the protein with a dextran layer. Bovine Hb and Hb nanoparticles have been successfully conjugated to oxidized dextran in preliminary studies.[14,15] Dextran-coated bovine Hb showed similar oxygen carrier properties as the unmodified Hb, with a slightly decreased autoxidation rate.[16] Dextrans below 50 kDa MW are also correlated to a reduced rate of elimination in the body, which could potentially improve the half-life of the dextran conjugated HBOCs.[17] The purpose of this work was to determine a protocol for conjugating oxidized dextran (Odex) to LtEc, forming a new HBOC. A successful conjugation would increase the size and molecular weight of LtEc due to the coating material while retaining oxygen carrier properties. To study properties of the resulting molecule compared to native LtEc and other modified LtEc molecules, characterization methods from the literature were used to study the new material. A one-pot synthesis was developed from previous methods for the conjugation reaction and the resulting Odex-LtEc product was analyzed to determine the effectiveness of the synthesis and properties of the product.[11,12]

3 Methods

LtEc purification

The method developed by Salva et al. produced ultrapure LtEc protein via tangential flow filtration (TFF).[11] This protocol was used to isolate LtEc from whole worms. LtEc was concentrated to 50-70 mg/mL in 0.1 M phosphate buffered saline (PBS, pH 7.4) and was stored at -80 °C to retain its properties before further steps.

Preparation of oxidized dextran & aldehyde activity assay

To prepare Odex, 0.36 grams of dextran (40 kDa, Millipore Sigma) was dissolved in deionized (DI) water to a concentration of 50 mg/mL. Sodium periodate was added at a molar ratio of 1:1 periodate to glucose subunit and the solution was stirred in a covered glass vial for 1 hour. Odex product was isolated by dialyzing the solution in a cellulose tube (8 kDa) for ~48 hours against water, then lyophilized to produce solid crystals.

Aldehyde activity of the lyophilized Odex was confirmed by reaction with 2,4-Dinitrophenylhydrazine (DNPH).[15] Odex crystals were reconstituted to 20 mg/mL in DI. From this solution, 100 µL sample were added to 500 µL DNPH solution (0.1 M in 70 mL ethanol, 20 mL water, and 15 mL sulfuric acid), with a control of 100 µL water in 500 µL DNPH solution. After centrifuging the samples at 2000g for ~3 minutes to sediment the yellow precipitate, the supernatant was collected. Absorbance of the supernatant at 360 nm was determined with a UV-visible spectrophotometer (HP8452A, Olis Inc., Bogart, GA). The difference in DNPH between the control and sample was calculated using the extinction coefficient of 22,000 M^{-1} cm^{-1}.[18] Since the reaction between DNPH and aldehydes is 1:1, the molarity of consumed DNPH was equal to the concentration of aldehyde groups present in the Odex sample.

$$Aldehydes\ per\ Odex = \left(\frac{Abs_{sample} - Abs_{control}}{22{,}000\ (M * cm)^{-1}}\right) * \frac{D_{sample}}{M_{Odex}}$$

Where D_{sample} is the dilution factor of the sample in the cuvette, and M_{Odex} (mol/L) is the molarity of the Odex in the original sample.

Figure 1: Control (L) and sample (R) in DNPH solution, before centrifugation. Yellow precipitate forms upon reaction of DNPH with aldehydes.

Figure 2: Control (L) and sample (R) in DNPH solution after centrifugation. Absorbance measurements were taken for the supernatant.

Odex-LtEc synthesis

For each batch synthesis of Odex-LtEc, 0.36g Odex was rehydrated in PBS to 20 mg/mL. About 1.2 grams purified LtEc (previously frozen at -80 °C and thawed to 22 °C) were added to the Odex solution to achieve a ratio of ~15 Odex aldehydes to LtEc amine groups. The reaction proceeded at 22 °C for 4 hours with stirring. The reaction was then quenched by adding glycine (2M) at 10:1 the initial aldehyde concentration and sodium cyanoborohydride (100 mg/mL) at 4:1 to reduce the unstable imine bond in the product Odex-LtEc. Odex-LtEc was isolated via TFF with 10 volume exchanges of PBS across a 500 kDa polysulfone TFF cartridge (Spectrum Laboratories, Rancho Dominguez, CA), then was concentrated to 4 mg/mL and stored at -80 °C before analysis.

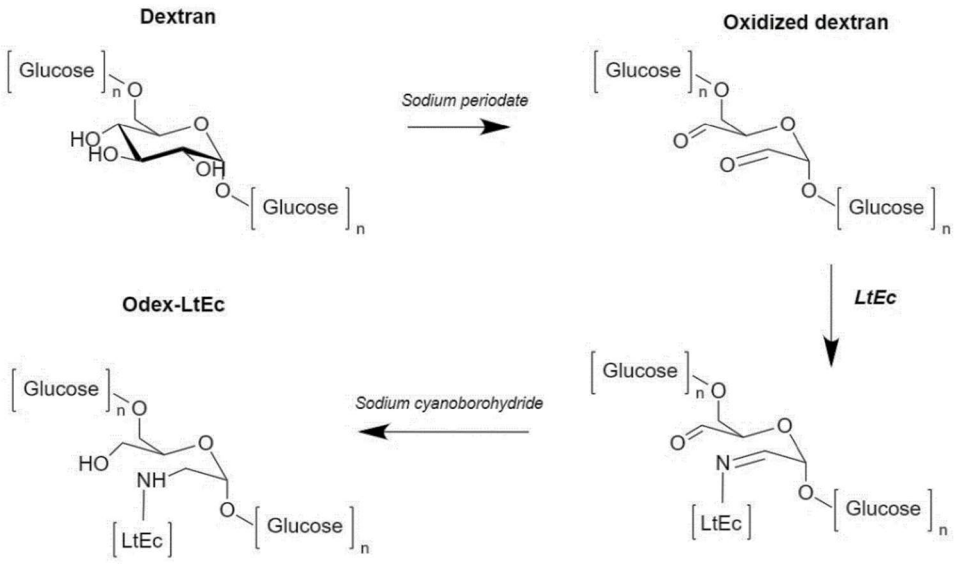

Figure 3: Functionalization of dextran, synthesis of Odex-LtEc, and imine quenching

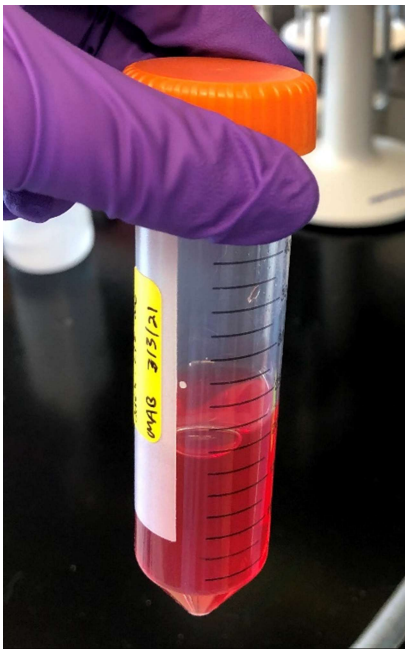

Figure 4: Odex-LtEc product (4 mg/mL.)

Characterization Studies

Size exclusion chromatography (SEC) was performed with a High-Performance Liquid Chromatography system (Dionex UltiMate 3000 UHPLC/HPLC, Thermo Fisher Scientific, Waltham, MA). Samples were diluted to 1 mg/mL, sterilized with 0.2 µM syringe filters, and separated via a mobile phase of 50 mM sodium phosphate buffer (7.4 pH) on an Acclaim SEC-1000 column (4.6 by 300 mm, Thermo Fisher Scientific, Waltham, MA). System parameters such as flowrate (0.35 mL/min) and absorbance detection (280 and 413 nm) were set using Chromeleon 7 software.

Samples were diluted to 1 mg/mL to perform zeta potential and diameter analysis. Zeta potential was determined with a Zeta-Pals Analyzer (Brookhaven Instruments, Holtzville, NY) and its corresponding software. Hydrodynamic diameter was determined via non-linear least squares fitting of values collected from a BI-200SM Goniometer (90° angle, 637 nm wavelength).

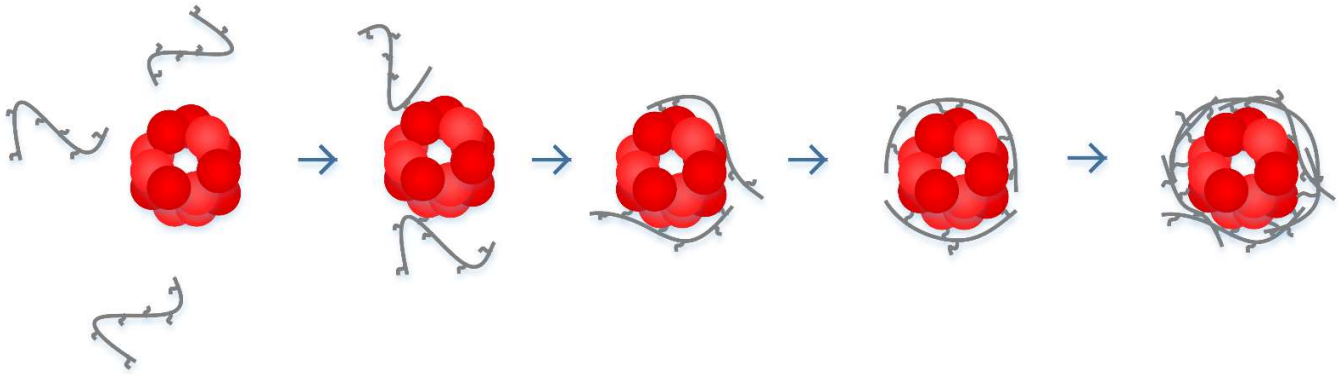

Figure 5: Depiction of the Odex-LtEc conjugation reaction over time. Functionalized dextran attaches to LtEc via aldehyde-amine bonds, forming a thin layer around the protein.

Oxygen Equilibrium Curves

Odex-LtEc samples were diluted to 1 mg/mL (60 µM heme basis) in HEMOX buffer (TCS Scientific, New Hope, PA). 20 µL of Additive A, 10 µL of Additive B, and 20 µL of antifoam (TCS Scientific) were mixed thoroughly with the solution. Oxygen equilibrium curves were generated for the solution at 37 °C using a HEMOX Analyzer (TCS Scientific, New Hope, PA). Samples were bubbled with air to reach 142 mmHg partial oxygen pressure, then bubbled with nitrogen to deoxygenate. Absorbance readings were collected as the partial O2 pressure dropped. The results were fit to the Hill equation to determine the n and P_50 values.[19]

$$Y = \frac{A_{sample} - A_{pO2=0}}{A_{pO2,max} - A_{pO2=0}} = \frac{{pO_2}^n}{{pO_2}^n + {P_{50}}^n}$$

Stopped-flow Rapid Deoxygenation Kinetics

Odex-LtEc samples were diluted to 12.5 µM heme in PBS. The deoxygenated buffer was prepared as 2 mg/mL sodium dithionite in deionized water. Absorbance readings were taken at 437.5 nm as the sample was rapidly mixed with the buffer in a microvolume stopped-flow spectrophotometer (Applied Photophysics Ltd., Surrey, UK). The rate of rapid oxygen offloading for Odex-LtEc at ambient temperature (22 °C) was determined by fitting an exponential decay model to the absorbance data.

Autoxidation Rate

Odex-LtEc samples were diluted (60 µM heme in PBS) and held at 37 °C using a recirculating water bath. Absorbance spectra from 300 to 700 nm were taken with a UV-visible spectrophotometer each hour for 24 hours. Concentrations of oxygen-carrying LtEc (oxyLtEc) and oxidized LtEc (Fe^{3+} state, metLtEc) were quantified for each time point based on spectra of the pure species for native LtEc. The relative remaining oxyLtEc was fitted to a first-order kinetics model to determine a rate constant for the oxidation reaction.

4 Results & discussion

Efficacy of synthesis protocol

Odex reactivity after lyophilization was quantified as 75 aldehydes per Odex molecule. An excess of Odex was added to the native LtEc for conjugation such that the aldehyde to amine ratio was approximately 15:1. The Odex-LtEc had a significantly larger hydrodynamic diameter of 44 nm and shorter SEC elution time of 7.8 minutes, indicating a molecular weight greater than LtEc. The zeta potential of both native and Odex-LtEc were negative, indicating both groups would be compatible with the predominantly negative surface potential in the circulatory environment. A significant shift in zeta potential between Odex-LtEc and LtEc further suggests the surface of the protein was altered by the addition of Odex.

Table 1: Summary of results for Odex-LtEc characterization

	Diameter [nm]	*Elution time [min]*	*Zeta potential [mV]*	*O2 P50 [mmHg]*	*Cooperativity (n)*	$k_{oxidation}$ *(1/hr)*	$k_{offloading}$ *(1/s)*
LtEc[12]	28.20 ± 9.41	8.113 (~3.6 MDa)	-30.4 ± 2.3	24.86 ± 2.84	2.92 ± 0.39	0.004 ± 0.001	29.19 ± 0.04
Odex-LtEc	44 ± 3.16 *	7.808 ± 0.0108 *	-18.89 ± 4.00 *	10.827 ± 1.379 *	1.564 ± 0.181 *	0.0537 ± 0.021 *	23.71 ± 1.44 *
PEG-LtEc (40% PEGylation)[12]	36.00 ± 8.93	7.150	-12 ± 1.6	19.64 ± 1.12	1.43 ± 0.18	20.36 ± 0.22	0.013 ± 0.003

*p<0.05 in a t-test with average LtEc value

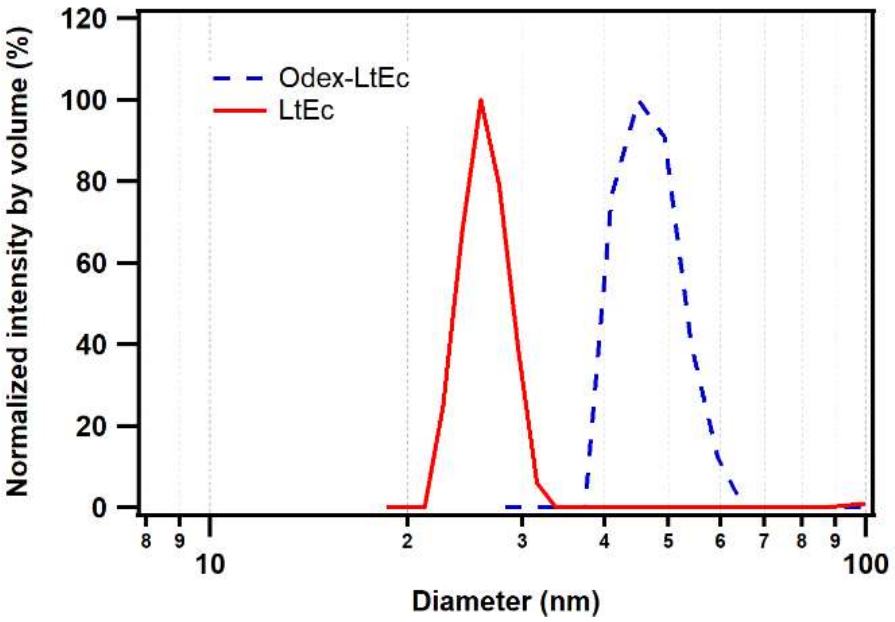

Figure 6: Hydrodynamic diameter measurements from dynamic light scattering. Odex-LtEc has a larger diameter (44 nm) than LtEc (28 nm).

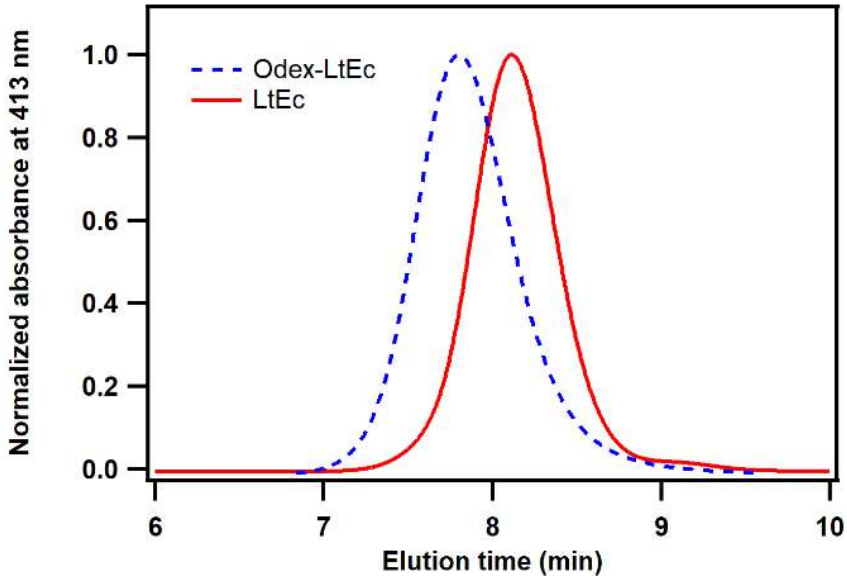

Figure 7: Elution time from a SEC column. LtEc takes longer to elute (8.1 min) than Odex-LtEc (7.8 min).

In vitro assays and HBOC properties

Odex-LtEc had a lower P_{50} and cooperativity than native LtEc at 37 °C. This aligns with prior results of LtEc coated with polyethylene glycol, as well as Hb conjugated to dextran.[20,21] The boundary layer of the surface coating may act as a positive diffusive barrier, allowing the heme groups to saturate with oxygen at lower partial oxygen pressure. The surface coating can also restrict movement, lowering the allosteric effects of the multiple heme groups within a hemoglobin molecule as indicated by reduced

cooperativity. The greater oxygen affinity predisposes Odex-LtEc as suitable for delivering oxygen under hypoxia (<10 mmHg pO_2).

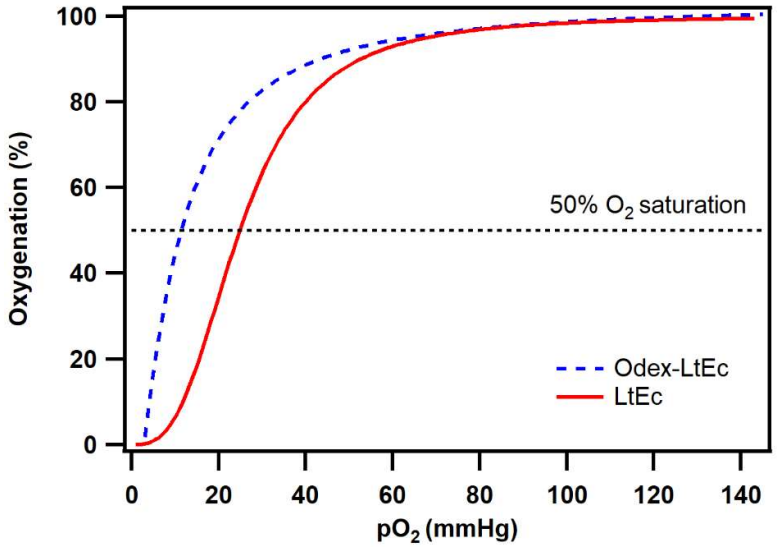

Figure 8: Oxygen binding equilibria at 37 °C fitted to the Hill equation. Cooperativity and P50 (10.8 mmHg) of Odex-LtEc are lower than LtEc (24.9 mmHg).

The coefficient in the exponent term of the model fitted to the stopped-flow absorbance data corresponded to the oxygen offloading rate constant, $k_{offloading}$ (s^{-1}). For Odex-LtEc, $k_{offloading}$ was significantly less than that of native LtEc, indicating Odex-LtEc releases oxygen more slowly. This is likely due to the steric hindrance of Odex polymers around the heme pockets, where oxygen binding and offloading take place. Note that the initial slow absorbance increase from t = 0 to 0.05 seconds (for LtEc) was eliminated to the curve fitting as this was observed due to the initial elimination of residual oxygen from the buffer and not related to the offloading reaction mechanism. Despite Odex-LtEc having a slower offloading rate than native LtEc, the oxygen offloading rate of LtEc is still faster than for human red blood cells (5 s^{-1}), so the offloading rate is sufficient for oxygen delivery in circulation.[22]

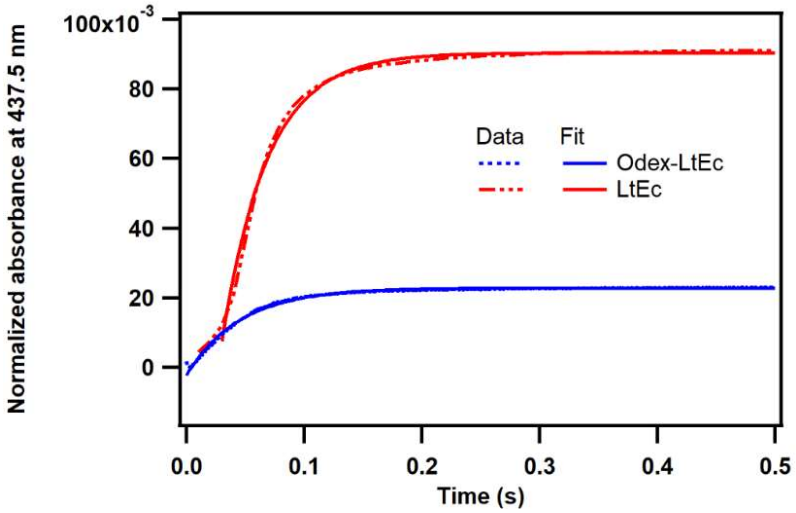

Figure 9: Rapid deoxygenation kinetics at 22°C. LtEc offloads oxygen at 29 s^{-1}, while Odex-LtEc offloads oxygen at 24 s^{-1}.

The autoxidation rate of native LtEc is much lower (0.004 hr^{-1}) than human Hb (0.033 hr^{-1}), indicating a greater ability for LtEc to stay in the reduced Fe^{2+} state at the heme iron.[9] Once metHb (Fe^{3+}) forms via oxidation, the Hb molecule is unable to carry oxygen. Thus, a low rate of spontaneous autoxidation is a desirable feature of HBOC candidates. Although Odex-LtEc showed higher rate of autoxidation at 37 °C than native LtEc, it not significantly different than the rate for human Hb (0.033 hr^{-1}).[12] PEG-LtEc and other modified HBOCs show increased autoxidation rates as well.[12] Furthermore, LtEc has been stored for long periods of time in buffers containing certain stabilizing agents and has the potential to be reduced in the blood due to the presence of antioxidant species such as ascorbic acid.[23,24] Since the autoxidation study was performed in phosphate buffered saline, none of these agents were included, and thus the autoxidation rate *in vivo* may be lower for Odex-LtEc than shown here.

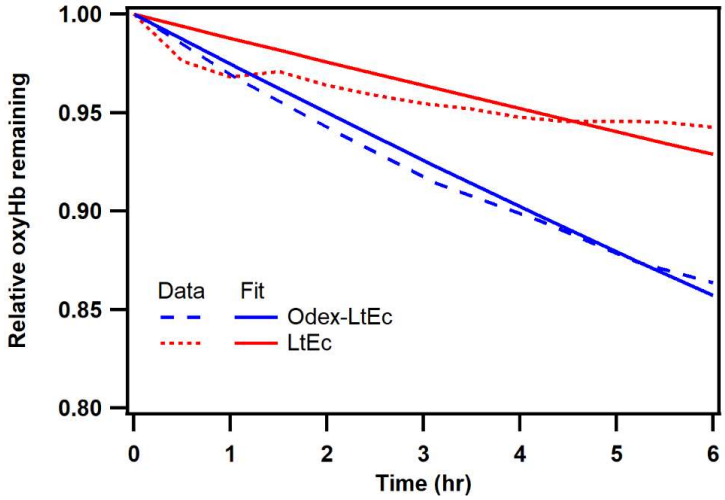

Figure 10: Autoxidation kinetics at 37°C. LtEc has an autoxidation rate constant of 0.004 hr^{-1} while the rate constant for Odex-LtEc is approximately 0.054 hr^{-1}.

5 Conclusions & future work

In summary, Odex-LtEc was synthesized from ultra-pure LtEc and lyophilized oxidized dextran. A one-pot reaction of the redissolved dextran with LtEc produced a product with a larger diameter and shorter elution time, indicating a significantly larger size than the native LtEc protein. The reaction protocol described here thus proves effective for some level of conjugation to LtEc, although modifications may be made in the future to pinpoint a ratio of reactants for optimal properties of the product. The oxygen-binding P_{50} and cooperativity of the Odex-LtEc product was lower than the native protein. The autoxidation rate at 37°C was elevated and the oxygen offloading rate was lower than the native protein due to surface coating interactions. The differences between Odex-LtEc and native were similar to those observed for PEG-LtEc. The Odex-LtEc product should next be evaluated in animal models to determine effects *in vivo*. Additionally, the protocol for making the Odex-LtEc product should be evaluated at different ratios of Odex and reaction time to determine how these parameters affect the final product. Additional autoxidation studies may also be performed to more accurately mimic circulatory conditions *in vitro* or to determine the impacts of buffer solution on oxidation kinetics for long-term storage.

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